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ORIGINAL PAPER

Genetic composition, origin and conservation of loggerhead sea turtles (*Caretta caretta*) frequenting the French Mediterranean coasts

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Abstract

This study aims to characterise the genetic structure and composition of 245 individuals of loggerhead sea turtles collected from stranding and bycatch events along the French Mediterranean coasts (Gulf of Lion, Provence and Corsica). We obtained sequences of the mitochondrial control region for two fragments (683 bp and 241 bp for 170 and 51 individuals, respectively). The analysis of the long fragment revealed that 163 samples (95.9%) are attributed to the haplogroup II (mainly Mediterranean) whereas only seven individuals are included in haplogroup IB (Atlantic Ocean). The mixed stock analysis performed on the same dataset indicated that the biggest rookeries from the eastern Mediterranean mainly contributed to the French stock, with major contributions being from Greece (36% and 56% for adults and juveniles, respectively), Crete (12% and 18%) and Western Turkey (14% and 4%). The thirteen microsatellite nuclear markers that have been analysed for 81 specimens did not reveal much genetic structure within sampled individuals, thus suggesting that the studied individuals could belong to the same genetic group. The microsatellite analyses revealed however that the nine individuals issuing from two nests sampled in Gulf of Lion and Provence are clearly differentiated from the remaining samples, thus suggesting a long-distance colonisation of the western Mediterranean. Our results allowed addressing the question of loggerhead turtle conservation in the western Mediterranean basin that until now is not considered as a Management Unit despite high densities of juveniles and recent nesting observed on French, Italian and Spanish coasts.

Keywords Western Mediterranean Sea · Mitochondrial DNA · Microsatellite DNA · Genetic structure · Mixed stock analysis · Conservation genetics

Introduction

The loggerhead sea turtle (*Caretta caretta*), the most abundant sea turtle in the Mediterranean Sea (Broderick et al. 2002), is a highly migratory animal living in temperate and sub-tropical waters in all ocean basins (Dodd 1988). *Caretta caretta* shows a complex life cycle involving series of habitat shifts during their lifetimes (review in Bolten and Witherington 2003; Casale and Tucker 2017). After hatching on land, juveniles are transported by sea surface currents and discover the great ocean spaces in search of pleasant temperature and food. As sub-adults, they frequent feeding areas in coastal habitats whereas adults travel hundreds of kilometres between their feeding ground and breeding site (reproduction every 3 to 5 years, see Bolten and Witherington 2003). In this context, this slow maturating species is threatened by numerous and strong anthropogenic pressure. Intentional fishing, incidental captures (bycatch), collision

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with boats, interactions with debris (ingestion, entanglement) and habitat degradation are the main factors responsible of the general decline of populations (Margaritoulis et al. 2003). In this regard, the Mediterranean Sea is one of the most important hotspots for sea turtle bycatch (Casale and Margaritoulis 2010; Splendiani et al. 2017) which concern juveniles as well as adults.

The complex features characterizing the loggerhead sea turtles (large distribution and dispersal, the complexity of life cycle and diversity of threats) make adapted measures of protection challenging to implement and requiring the integration of diverse information. Wallace et al. (2010) conducted a multi-scale study including multiple tools and techniques to define Regional Management Units (RMU) for several marine turtles at a worldwide scale. For the loggerhead turtle, this study identified the Mediterranean Sea as one RMU. Later, Shamblin et al. (2014) recognized additional structuring in defining seven MUs on the basis of genetic structure and gene flow among 17 rookeries of the eastern Mediterranean Sea.

The main nesting sites of *C. caretta* in the Mediterranean Sea are located in the eastern basin (Cyprus, Greece, Turkey, Syria and Libya; Casale and Margaritoulis 2010). Females usually return to the same nesting beach in successive nesting seasons and they also return to their natal beach to nest (see Bolten and Witherington 2003). This behaviour called "philopatry" or "natal homing" has consequences as evolutionary drivers as it can conduct to the formation of genetic structure and reproductively isolated matrilines (Bowen et al. 2005). However, it has been shown that females nesting in the north-western Atlantic have a remigration rate close to 70%, which means that a significant proportion of nesting females are not strictly philopatric and lay their clutches in other nesting beaches (Richardson et al. 1978). Moreover, there is a distinction between nesting and feeding turtle aggregations. The benthic habitats, used as foraging areas by sea turtles, are referenced in both oriental and occidental Mediterranean basins (Laurent and Lescure 1994). These feeding grounds that are of particular importance for juveniles bring together individuals of different origin (from Mediterranean and Atlantic rookeries, see Casale and Tucker 2017), with likely consequences on the expected genetic composition of both groups. Moreover, incidences are also anticipated according to the sea turtle developmental stage because adults are likely representing Mediterranean residents whereas more geographical mixing is expected for juveniles.

The genetic characterization of loggerhead sea turtles has been mainly conducted through the analysis of mitochondrial DNA (mtDNA) control region sequences. As maternally inherited, this marker gives information about the genetic architecture of loggerhead populations over both contemporary and evolutionary timescales. Furthermore, because most females are supposed to return to their rookery of origin, each nesting population should possess a genetic signature in terms of female transmitted mtDNA. This genetic marker has proven to be useful to identify the genetic composition of different nesting areas and thus to determine which sea turtle rookeries contribute to a particular feeding ground (Bass et al. 2004). Indeed, mtDNA data resolved nesting habitats at the Atlantic and Mediterranean scales, which differed significantly in haplotype composition and frequency (Bowen and Karl 2007). By contrast, the analysis of nuclear DNA microsatellites, thus biparentally inherited markers, usually revealed no genetic structure between rookeries or foraging areas (Bowen et al. 2005, Monzón-Argüello et al. 2010, Yilmaz et al. 2011, Garofalo et al. 2013), a result that is usually attributed to male-biased gene flow. By contrast, the study of Clusa et al. (2018) based on 15 microsatellites and 8 nesting grounds from the Eastern Mediterranean identified 5 clusters thus suggesting that widespread male gene flow could have been overstated. At the Mediterranean scale, molecular studies concerned nesting rookeries and foraging grounds in different places (eastern and central Seas, Spanish and Algerian basins; Carreras et al. 2007; Garofalo et al. 2013; Clusa et al. 2014). These studies indicated that loggerhead sea turtles frequenting the Mediterranean have different geographical origins (not only from different Mediterranean places but also from the Atlantic Ocean). Moreover, turtles are not uniformly distributed in the different basins as exemplified by the turtles of Atlantic origin that are predominantly found in the Algerian basin (Clusa et al. 2014). However, if most Mediterranean areas have been considered until now no study has concerned sea turtles that can be found off the French coastline.

The purpose of this study was to trace the diversity and origin of adults and juveniles of loggerhead turtles (*C. caretta*) stranded or accidentally caught on the French Mediterranean coasts of the Gulf of Lion, Provence and Corsica on the basis of mitochondrial and nuclear DNA. To this end, we analysed sequences of the mtDNA control region (241-bp and 683-bp) as well as 13 microsatellites loci developed by Shamblin et al. (2007) and Monzón-Argüello et al. (2008). Specifically, the objectives were to (1) characterize the population structure and evolutionary history of loggerheads found on the French Mediterranean coasts by describing the mtDNA haplotypes and nuclear microsatellites composition, (2) infer the origin and assemblage of French sea turtles using a mixed-stock analysis (MSA), and (3) draw some conclusions in terms of conservation, notably with respect to juveniles and sporadic nesting events recently occurring on the sandy beaches of the Gulf of Lion and Provence.

Materials and methods

Sampling and molecular analyses

Loggerhead turtles are strictly protected in France (national decree of October 14, 2005) and the sample collection was allowed by the decree of December 31, 2012 on the renewal of the environmental protection approval of the Société Herpétologique de France (SHF), and the decree of October 24, 2016 on the collection of biological data in the event of sea turtle stranding or bycatch on French metropolitan coasts (NOR: DEVL1500415N) for the mainland sea turtle observatory program of the National Museum of Natural History (MNHN).

Blood and tissue samples from 245 loggerhead turtles or eggs were collected from 1989 to 2018 on the Mediterranean coastline of three regions (Fig. 1, Online Resources 1 and 2): Gulf of Lion (from the Spanish-French border to western Var: departments of Pyrénées-Orientales, Aude, Hérault, Gard, Bouches-du-Rhône, western Var), Provence (department of Alpes-Maritimes and eastern Var) and Corsica (departments of Haute-Corse and Corse du Sud).

Turtle specimens (see Online Resource 1 for characteristics of individuals) were caught accidentally or found stranded along the Mediterranean coastline whereas eggs, embryos or dead hatchlings ($N = 14$) were collected in three nests from three localities (see details below). The sex of dead individuals was determined by the observation of gonads during necropsies. For live turtles, adult males and females were identified with secondary sexual characters (*i.e.* size and shape of the tail). Each turtle was measured (minimum curved carapace length; CCLmin ± 1 cm). The mean size of males was 56.2 ± 0.21 cm (min = 31.5; max = 79.0 cm, $N = 21$) and the mean size of female was 58.6 ± 0.13 cm (min = 30.0; max = 76.0 cm, $N = 37$). The 156 individuals for which the sex was undetermined had a CCLmin of 43.25 ± 0.05 cm (min = 27.0; max = 75.0 cm) and were classified as juveniles. Twelve individuals measured less than 10 cm (7.69 ± 0.04 cm, min = 5 cm; max = 10 cm) were also considered as juveniles. For 19 individuals, no information was available about sex or body size (Online Resources 1).

Blood samples ($N = 218$ with 46 samples from dead specimens and 172 from live animals) were collected from the dorsal cervical sinus and stored in 70% ethanol at 4 °C. Tissue samples (*e.g.* 1 cm^3 of muscle from the pectoral region or front flippers) were collected from dead individuals and stored at − 20 °C. Tissue samples from nests consisted of eggshell fragments preserved in 10° formalin ($N = 4$; nesting

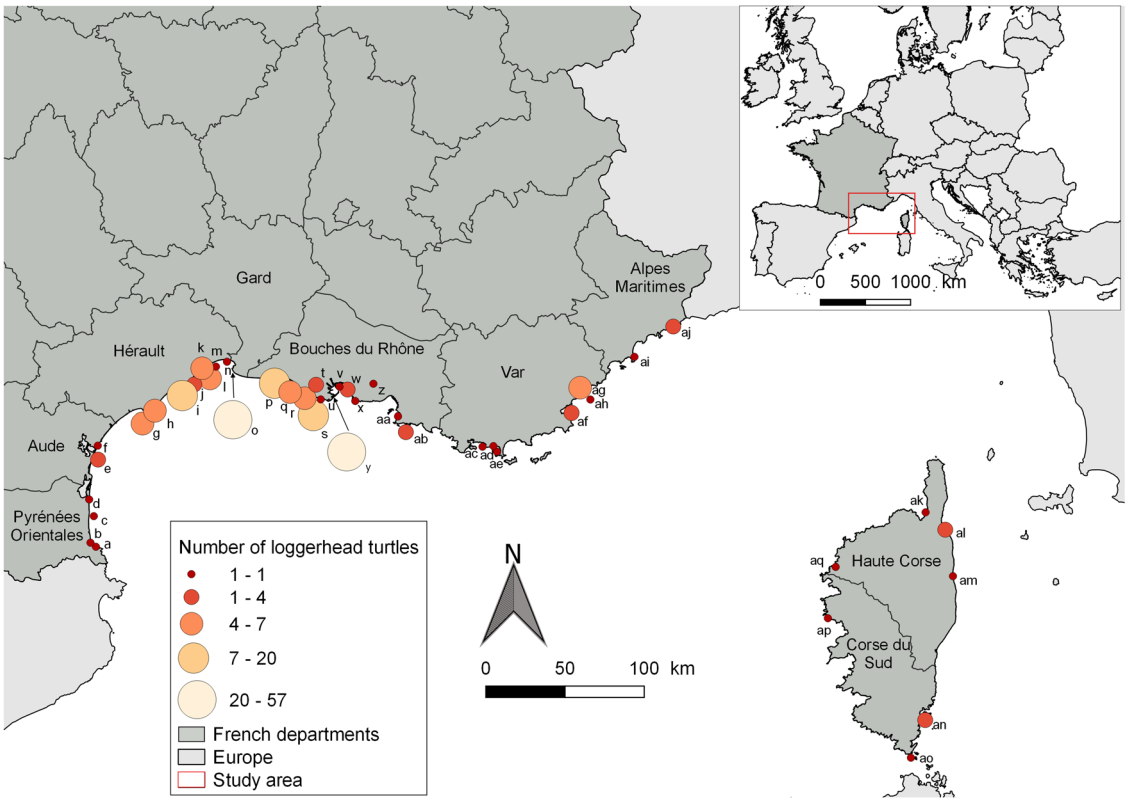


Fig. 1 : Sampling locations along the Mediterranean French coastline. Letters corresponds to localities provided in Online Resource 2

event in Saint-Tropez [af in Fig. 1], Var in July 2006), whole eggs preserved in 70% ethanol ($N = 5$; nesting event in Saint-Aygulf [ag], Var in July 2016) and dead hatchlings or embryos kept at − 20 °C ($N = 5$; nesting event in Villeneuve-lès-Maguelone [k], Hérault in October 2018). DNA was extracted from blood samples using the REDExtract-N-Amp Plant Kit (Sigma) or with the Qiagen DNeasy Blood and Tissues kit (QIAGEN®) for tissue samples following the manufacturers' instructions.

Mitochondrial DNA

A fragment of non-coding mtDNA control region was amplified by Polymerase Chain Reaction (PCR) using the primers LCM15382 (5′-GCTTAACCCTAAAGCATTGG-3′) and H950 (5′-AAACCCCTAAATCCGAGAC-3′) (Abreu-Grobois et al. 2006) referred to as "long sequence" (~ 800 bp) or with primers LCM15382 and CC443 (5′-TGATCTATTCTGGCCTCTG-3′; Shamblin et al. 2014) referred to as "short sequence" (~ 380 bp). PCRs were carried out in 20 µl volume containing 10 µl of ReadyMix™ Taq Sigma (2X), 1 µl of each primer at 10 pM (final concentration at 0.5 pM), 7 µl of pure water and 1 µl of DNA diluted at 1/30 dilution or pure when DNA was degraded (*i.e.* blood samples from autopsy). PCR cycling parameters were as follows: 95 °C for 3 min; 34 cycles at 95 °C for 30 s, 55 °C for 1 min, 72 °C for 30 s; and a final extension of 72 °C for 10 min. Amplification products were separated by 1% agarose gel electrophoresis in a 0.5X TBE buffer and visualised with ethidium bromide in UV light. The DNA concentration of each sample was evaluated after electrophoresis on agarose gel by comparison with a standard molecular weight marker (100pb PCR DNA ladder, Fisher Scientific). The diluted PCR products were sent to Eurofins Genomics (Ebersberg, Germany) for purification and Sanger sequencing. Electropherograms were visually checked and corrected using the program CODONCODE ALIGNER Version 5.1.5 for Windows (CodonCode Corporation, Dedham, MA, USA) and a consensus sequence was compiled.

Microsatellites

Thirteen microsatellite loci previously described for *C. caretta* (Cc-13, Cc-17, Cc-25 and Cc-28 from Monzón-Argüello et al. 2008; Cc1B03, Cc1G02, Cc2G10, Cc5H07, Cc5C08, Cc1F01, Cc7E11, Cc1G03 and Cc7C04 from Samblin et al. 2007) were used (see Online Resource 3 for characteristics of loci). Each locus was amplified separately using PCR cycling parameters as follows: 95 °C for 15 min; 30 cycles at 94 °C for 30 s, 60 °C or 61 °C for 1 min 30 s (61 °C for the 9 loci from Samblin et al. 2007 and 60 °C for the 4 loci from Monzón-Argüello et al. 2008), 72 °C for 1 min; and a final extension of 60 °C for 30 min. After dilution of PCR products according to the amount of DNA, genotyping was performed on a 24-capillary sequencer (3500XL DNA Analyzer, Applied Biosystems) at the platform « Génotypage-Séquençage» of the labex « Centre Méditerranéen de l'Environnement et de la Biodiversité» (Montpellier, France). The generated microsatellite profiles were visualized using GeneMapper Version 5.0 (Applied Biosystems) and the genotype was determined after two readings by two independent people.

Population genetic analysis

Mitochondrial DNA

Sequences were aligned, edited and compared to previously described haplotypes using the BIOEDIT program Version 7.2.5 (Hall 1999). Sequences were assigned to haplotype designations according to the nomenclature rules published at the Archie Carr Center for Sea Turtle Research (ACCSTR) database (http://accstr.ufl.edu/). All haplotypes previously reported from the Atlantic, Mediterranean and Northern Pacific were included in the analyses (Nishizawa et al. 2014; Shamblin et al. 2014). Relationships between haplotypes were visualized using the software Network (Bandelt et al. 1999) with the median-joining algorithm to allow multi-state data. Haplotype distribution maps were generated with QGIS Version 3.2.1-Bonn (http://qgis.osgeo.org"). DnaSP Version 5.10.01 (Librado and Rozas 2009) was used to calculate mitochondrial diversity indices including haplotype and nucleotide diversities, number of haplotypes and polymorphic sites.

Past population dynamics through time was inferred using the Bayesian skyline plot (BSP) model (Drummond et al. 2005) implemented in Beast V.1.10.4 (Suchard et al. 2018). BSP analysis allows describing the change in effective population size (Ne) in the course of time (the timescale being given by the mutation rate of the sequences analysed). The demographic analysis was performed on haplogroup II using the Bayesian skyline as coalescent and the substitution model GTR + G + I applied to the non-coding control region dataset. Two molecular clocks models (Strict Clock and Uncorrelated Relaxed Clock) were tested using as prior the mean rate obtained by Duchene et al. (2012) for the control region of marine turtles, that is 3.24×10^{-3} substitution/site/10^6 years [95% HPD: 2.66–3.81]. Beast was run for 200 million generations sampled every 1000th to get ESS values > 200 for each parameter. Bayesian skyline plots (BSP) were built using Tracer v1.7.1 (Rambaut et al. 2018) using a 10% burn-in.

A Bayesian mixed stock analysis (MSA) was conducted to estimate the geographic composition of the individuals analysed using the haplotype frequency (long fragment) of 23 rookeries (10 Atlantic and 13 Mediterranean rookeries)

as a baseline (Shamblin et al. 2014, Splendiani et al. 2017, Tolve et al. 2018). MSA was performed with the package MIXSTOCK Version 0.9.5.1 (Bolker 2012) under RStudio Version 1.2.5042.

Microsatellites

The software MICRO-CHECKER v.2.2.3 (Van Oosterhout et al. 2004) was used to estimate null allele frequencies for each locus. The number of alleles (k), observed (Ho) and expected (He) heterozygosity, fixation index (F), Hardy–Weinberg equilibrium (*p*HWE) deviation and the inbreeding coefficient (Fis) were calculated with GenAlEx v.6.502 (Peakall and Smouse 2012). To investigate the genetic structure, we used the software STRUCTURE version 2.3 (Pritchard et al. 2000), which uses a Bayesian clustering method to estimate the most likely number of populations (K) without a priori information. Ten runs were carried out by setting the number of clusters (K) from 1 to 10 (number of populations) with a burn-in length of 350,000 followed by 2,000,000 Markov Chain Monte Carlo (MCMC) iterations. The determination of the most likely number of genetic groups was estimated from the method of Evanno et al. (2005) using STRUCTURE Harvester (Earl and vonHoldt 2012). A Principal Component Analysis (PCA) was done to represent the general organization of the global genetic variability of the sampling considered. PCA was conducted using the RStudioVersion 1.1.456, package adegenet and ade4 (Dray and Dufour 2007; Jombart 2008) to rank and visualize individuals depending on their genetic proximity.

Results

Genetic diversity and structure based on mtDNA

Haplotypes of the studied individuals

Of the 245 samples, we obtained long fragments for 170 individuals (30 adults, 132 juveniles and 8 unknowns). Negative PCRs for the long sequence (N = 75) were then amplified for the short fragment, leading to 51 supplementary sequences. Among the 24 negative samples, 11 (45.83%) were from necropsies. Because some sequences were shorter than others, they were truncated to obtain the same length for all individuals, thus resulting in the long (683 bp) and short (241 bp) alignments. We observed 34 variable sites for the long sequences and 13 for the short ones. For the short fragment (51 sequences), three haplotypes were identified. One haplotype, CC-A2 in the ACCSTR database, was by far the most common with 86.27% followed by haplotype CC-A3 represented by 11.76% of individuals. Haplotype CC-A20 was observed for only one individual. These three haplotypes are shared by Mediterranean and Atlantic rookeries (Saied et al. 2012; Shamblin et al. 2014). For the long fragment, 12 haplotypes were discovered and all of them have already been described in previous studies. The most abundant haplotype (81.7% of individuals) corresponds to the haplotype CC-A2.1 in the ACCSTR database. This haplotype was found in all the individuals (eggs and dead hatchlings) from the three French nesting sites. Haplotype CC-A3.1 was the second most common haplotype (7%) followed by haplotype CC-A.1.1 (2%) and haplotype CC-A2.8 (1.8%). Haplotypes CC-A1.3, CC-A6.1, CC-A20.1 and CC-A32.1 were represented by 2 individuals and haplotypes CC-A1.2, CC-A2.9, CC-A10.4 and CC-A31.1 by only 1 individual.

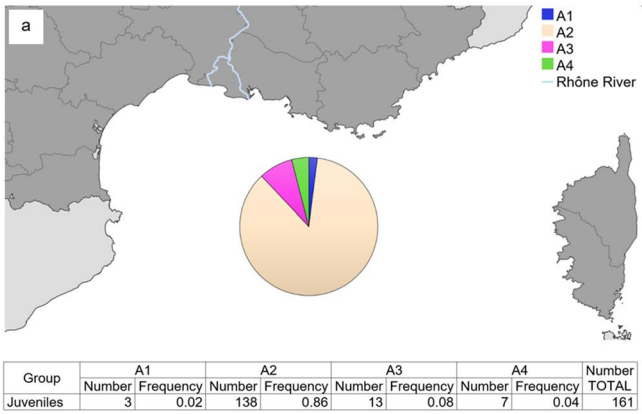
Nine individuals showed haplotypes exclusively described in Mediterranean rookeries (CC-A2.8, CC-A2.9, CC-A6.1, CC-A31.1 and CC-A32.1) whereas five individuals (all juveniles) had haplotypes exclusively described in Atlantic rookeries (CC-A1.1, CC-A1.2). The large majority of individuals ($N = 156$; 91.8%) carried the haplotype CC-A2.1 that is shared between Mediterranean and Atlantic rookeries including Cape Verde (Clusa et al. 2014). Figure 2 shows the frequency of the different haplotypes for adults and juveniles.

Network analysis

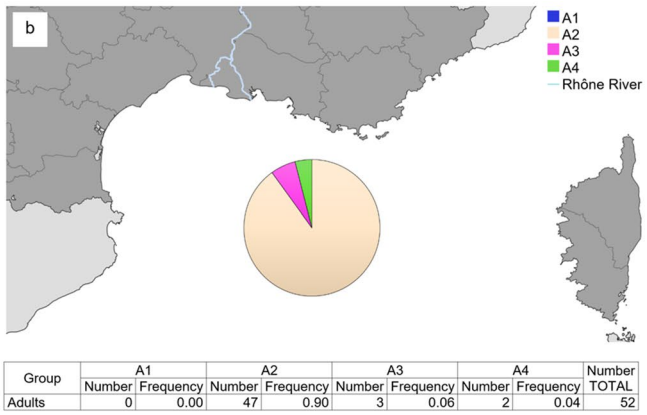
For the network analysis, 119 haplotypes previously published (Nishizawa et al. 2014; Shamblin et al. 2014) have been added to our dataset, thus representing 32 sequences for the short fragment and 87 for the long fragment.

The network performed on the long fragment (Fig. 3) allowed distinguishing three main haplogroups (nomenclature according to Shamblin et al. 2014) that mainly represented geographic groups: haplogroup IB included a majority of Atlantic haplotypes, haplogroup II encompassed haplotypes from the Atlantic Ocean and the Mediterranean Sea and haplogroup IA corresponded to haplotypes found exclusively in the Pacific Ocean. Haplogroups IA and IB are separated by 16 substitutions, haplogroups II and IA by 24 substitutions and haplogroup IB and II by 41 substitutions. In haplogroup II, haplotypes generally differed by a single mutation, unlike haplogroup IA and IB where haplotypes were more divergent. Among our 170 individuals, seven individuals are part of the haplogroup IB (4.1%) *versus* 163 for the haplogroup II (95.9%) and none for the haplogroup IA. In the haplogroup II, 140 individuals have the same and the most common haplotype CC-A2.1 (85.9%).

Two haplotype networks (Fig. 4) were then built on our long fragment dataset split into juveniles ($N = 132$) and adults ($N = 30$). For the juveniles (Fig. 4a), 10 haplotypes were recovered on which only two haplotypes represented



Group	A1		A2		A3		A4		Number
	Number	Frequency	Number	Frequency	Number	Frequency	Number	Frequency	TOTAL
Juveniles	3	0.02	138	0.86	13	0.08	7	0.04	161



Group	A1		A2		A3		A4		Number
	Number	Frequency	Number	Frequency	Number	Frequency	Number	Frequency	TOTAL
Adults	0	0.00	47	0.90	3	0.06	2	0.04	52

Fig. 2 Map of haplotype frequencies for **a** 161 juveniles and **b** 52 adults. Group A1 includes haplotypes CC-A1, CC-A1.1, CC-A1.2 and CC-A1.3. Group A2 comprises haplotypes CC-A2, CC-A2.1, CC-A2.8 and CC-A2.9. Group A3 includes haplotypes CC-A3 and CC-A3.1. Group A4 represents other haplotypes (CC-A6.1, CC-A10.4, CC-A20, CC-A20.1, CC-A31.1 and CC-A32.2) of low frequency

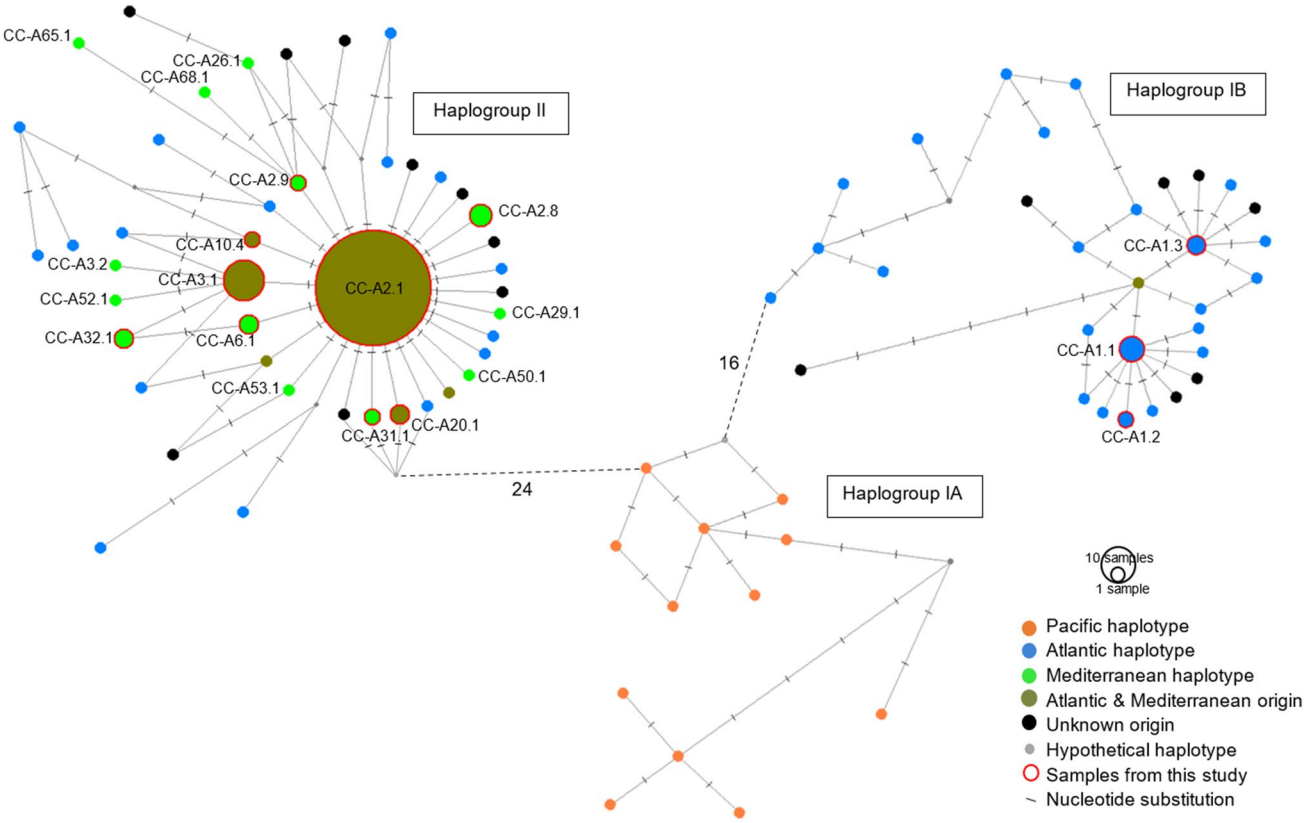


Fig. 3 Haplotype network obtained for the long fragment (683 bp) of the control region for our 170 samples of *Caretta caretta* added to the 87 haplotypes from Nishizawa et al. (2014) and Shamblin et al. (2014). Substitutions between haplotypes can be transitions, transversions or indels. Grey circles indicate unsampled or extinct haplotypes. Sizes of circles are proportional to sample sizes

by three individuals were included in the haplogroup IB. The remaining eight haplotypes corresponded to the haplogroup II among which CC-A2.1 was the majority haplotype (111 juveniles, *i.e.* 84.1%). Five haplotypes were observed for the 30 adults that all belonged to the haplogroup II (Fig. 4b) still with CC-A2.1 in the majority. This haplotype was detected in all departments along the French Mediterranean coastline and all localities except for five (Canet-en-Roussillon, La

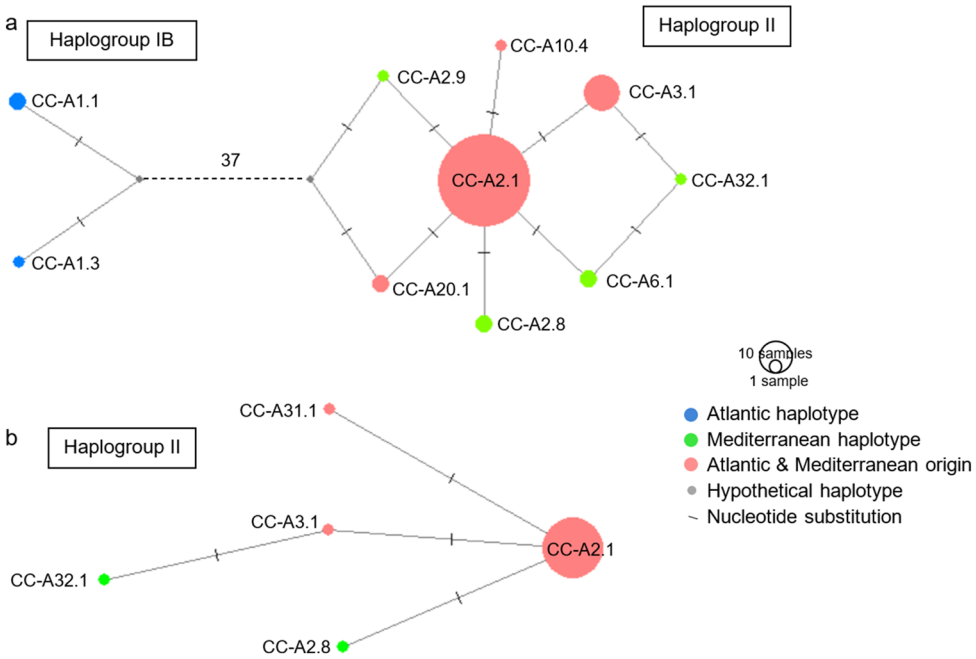


Fig. 4 Haplotype network obtained for the long fragment (683 bp) of the control region for **a** 132 juveniles and **b** 30 adults of *Caretta caretta*. Substitutions between haplotypes can be transitions, transversions or indels. Grey circles indicate unsampled or extinct haplotypes. Sizes of circles are proportional to sample frequencies

Grande Motte, Carqueiranne, Bonifacio and an unknown locality; see Online Resource 2) but only one or two samples were collected in these localities. The second most abundant haplotype (CC-A3.1) is represented by 12 samples from eight different locations. These two haplotypes are found in Mediterranean and Atlantic basins. The haplogroup IB is represented only by seven samples and six different locations (see Online Resource 2).

Genetic diversity indices

Genetic diversity indices were calculated for the short (241 bp) and long (683 bp) fragments as well as for the three haplogroups (Table 1). In all cases, the dataset included our samples (221 in total) combined to the 87 haplotypes described by Nishizawa et al. (2014) and Shamblin et al. (2014).

Calculations performed on the different datasets reflected the same trend *i.e.* the haplotype diversity (Hd) is higher for the long than for the short fragment. In all cases, haplogroups IA and IB are more diversified than haplogroup II in agreement with the network (Fig. 3). Haplogroup II is a star-like network showing a majority haplotype (CC-A2.1) from which numerous minority haplotypes are issued. Haplogroup II is also characterized by a lower nucleotide diversity (π) reflecting the fact that numerous haplotypes diverged from a single mutation from the majority haplotype. The nucleotide diversity is higher for the datasets including all haplotypes and for the long fragments whatever the haplogroup.

Demographic analysis

The demographic analysis was performed on the haplogroup II constituted 207 sequences of the long fragment. The comparison of the two molecular clocks tested identified the

Table 1 Genetic diversity indices for short (241 bp) and long (683 bp) sequences in different haplogroups including our samples combined to all haplotypes referenced by Nishizawa et al. (2014) and Shamblin et al. (2014)

Sequences analysed	*N*	*n*	Hd	*S*	*p*	π
Total haplotypes (short sequences)	253	32	0.42	30	30	0.0091
Total haplotypes (long sequences)	258	87	0.70	89	73	0.0151
Haplogroup IB (short sequences)	21	14	0.87	15	15	0.0078
Haplogroup IB (long sequences)	39	31	0.98	30	28	0.0060
Haplogroup II (short sequences)	232	18	0.32	17	17	0.0015
Haplogroup II (long sequences)	207	44	0.54	39	34	0.0012
Haplogroup IA (long sequence)	12	12	1.00	14	14	0.0075

N Total sample size; *n* Number of haplotypes; *Hd* Haplotype diversity; *S* Number of variable sites; *p* Number of segregation (polymorphic) sites; *π* Nucleotide diversity

strict clock as the best model over the uncorrelated relaxed clock (AICM = 8.55). The Bayesian Skyline Plot (Fig. 5) indicated a strong population expansion (increase of Ne) starting at about 130 kyrs [95% HPD: 90–180].

Mixed Stock Analysis

The Mixed Stock Analysis (MSA) was performed on our 170 sequences (30 adults, 132 juveniles and 8 unknown) of the long control region fragment. The analysis highlighted that loggerhead turtles found along the French Mediterranean coastlines mainly come from the Mediterranean (~ 89%) and to a lesser extent from Atlantic (~ 11%) rookeries (Fig. 6). Overall, the majority of the contribution comes from the eastern Mediterranean Sea: Greece (39.9%), Crete (12.3%), Western Turkey (12.3%), Dalyan rookeries in Turkey (5,8%), Lebanon and Israel (4,9%) and Calabria rookeries in Italy (3.1%). The contribution of the Atlantic Ocean is represented essentially by rookeries from Florida (8.8% among which Dry Tortugas with 4% and Canaveral National Seashore + Melbourne Beach with 3.2% are the most important). Finally, the contribution of other rookeries is negligible (less

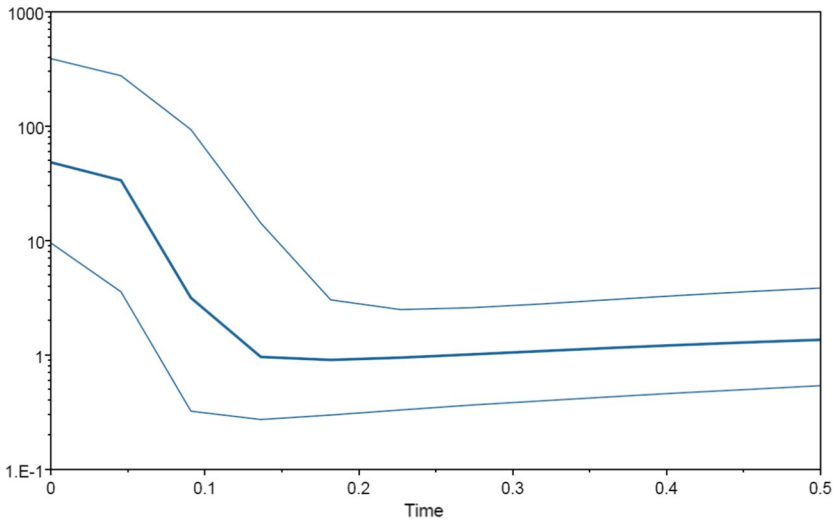


Fig. 5 Bayesian Skyline Plot obtained with Beast on haplogroup II (207 long sequences) showing an expansion phase starting at about 130 kyrs. X- and Y-axes represent the time (T in Myrs) and the effective population size (Ne in logarithm scale), respectively. The three lines represent the median estimate (dark blue) and the 95% highest posterior density (light blue)

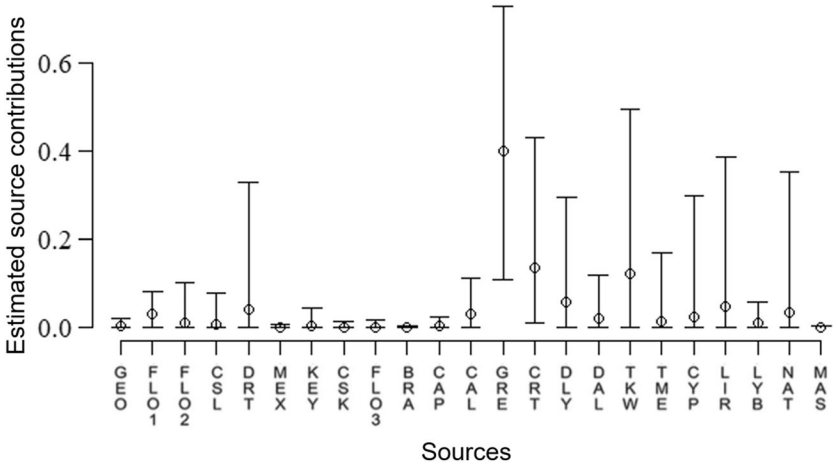


Fig. 6 Mixed Stock Analysis performed on 170 sequences of the long control region fragment. Estimated contributions and 95% confidence intervals of 23 loggerhead rookeries from the Atlantic Ocean and the Mediterranean Sea to the loggerhead turtles from the French Mediterranean coastline. Nesting areas: GEO (Cape Island, South Carolina + Ossabaw Island, Georgia), FLO1 (Canaveral National Seashore + Melbourne Beach, Florida), FLO2 (Juno Beach + Ft. Lauderdale, Florida), CSL (Cay Sal, Bahamas), DRT (Dry Tortugas, Florida), MEX (Isla Cozumel + Quintana Roo mainland, Mexico), KEY (Keewaydin Island, Florida), CSK (Casey Key, Florida), FLO3 (St. George Island + Cape San Blas, Florida), BRA (Sergipe + Bahia + Espírito Santo + Rio de Janeiro, Brazil), CAP (Boa Vista + Sal + Santa Luzia + Maio, Cape Verde), CAL (Calabria, Italy), GRE (Zakynthos Island + Kyparissia + Lakonikos, Greece), CRT (Rethymno, Crete), DLY (Dalyan, Turkey), DAL (Dalaman, Turkey), TKW (western Turkey), TME (middle Turkey + eastern Turkey), CYP (Alagadi + Akamas, Cyprus), LIR (El Mansouri, Lebanon + Israel), LYB (Sirte + Misurata, Lybia), NAT (Tongaland, KwaZulu-Natal, South Africa), MAS (Masirah, Oman)

than 5% such as Tongaland in South Africa with 3.4%) or undetected (such as for Cape Verde or Brazil). MSA performed only on the 132 juveniles (data not shown) have pointed out a major contribution from the Mediterranean Sea, especially from Greece (36.2%), Western Turkey (13.6%), Crete (11.6%) and Lebanon and Israel rookeries (8.2%). The contribution of the Atlantic Ocean is 8.5% with a majority from Dry Tortugas in Florida (3.1%) followed by Canaveral National Seashore and Melbourne Beach rookeries in Florida (2.6%). The results obtained only on the 30 adults (data not shown) highlighted Greece (55.8%), Crete (18.1%) and Western Turkey (3.9%) rookeries as major contributors to French feeding grounds whereas the Atlantic Ocean contributed for less than 6% with a majority from the Bahamas followed by Florida rookeries.

Genetic diversity and structure based on microsatellites

A subsample of 81 individuals from the French Mediterranean Coast was tested for population structure using 13 microsatellite loci. The sampling included individuals from the control region haplogroups IB and II (5 and 76 individuals, respectively). Moreover, all control region haplotypes evidenced (Fig. 4) as well as a maximum of localities (23 out of 43; Online Resource 1) were represented. The 13 microsatellites loci were polymorphic for the 81 samples analysed with a number of alleles ranging from 4 to 16 which leads to an average number of alleles per locus of 11.77. Analysis with the software MICRO-CHECKER indicated the possible presence of null alleles for three loci (Cc7C04, Cc-25 and Cc1B03) but the two last loci have an important ratio of missing data (see Online Resource 3). Observed and expected heterozygosity values (Ho and He, Online Resource 3) calculated for each locus ranged from 0.56 to 0.92 and from 0.67 to 0.90, respectively. Five loci showed a significant deviation from the Hardy–Weinberg equilibrium (pHWE < 0.05, Online Resource 3): Cc-25, Cc1B03, Cc7C04, Cc7E11, and Cc5H07.

A hierarchical analysis was conducted with STRUCTURE at several scales. A first analysis was run on the 81 individuals genotyped using a K variable value ranging from 1 to 10. The maximum likelihood and the delta (K) curve obtained with Evanno's method returned a maximum value of 2 clusters (Fig. 7a). This analysis mainly individualizes the nine individuals from the two spawning nests of Villeneuve-lès-M. and St-Aygulf (8 and 9 in Fig. 7b). The remaining 67 individuals of the haplogroup II (2 to 7 in Fig. 7b) appeared genetically mixed. To avoid a possible influence of sibling relatives, a second analysis was run with only one individual from each nest (74 individuals analysed). In this case, even if the delta (K) curve returned a maximum value of 2 clusters, the maximum likelihood is obtained for K = 1 and the barplot showed no population structure (Online Resource 4a). Moreover, as evidenced in Fig. 7b, there is no apparent difference between male and female (groups 3 and 4 for females *versus* groups 5 and 6 for males). We also performed STRUCTURE analyses in differentiating juveniles (59 individuals with only one sample per nesting site; Online Resource 4b) and adults (15 individuals; Online Resource 4c). In both analyses, two clusters are identified with the delta (K) curve but the barplots (data not shown) did not reveal any structuring among individuals. Another STRUCTURE analysis that was run with only eleven loci (*i.e.* without Cc-25 and Cc7C04 because of their high level of missing data) gave the same results.

A PCA was carried out on the 81 individuals genotyped for the 13 loci that have been split into four groups: the five samples from the haplogroup IB, the five individuals from the nesting event in Villeneuve-lès-M., the four samples from the nesting event in St-Aygulf and the remaining samples of the haplogroup II. The three first axes explained 17% of the variance. The first axis separates groups 2 and 3 whereas the second axis dissociates clusters 2 and 4. Finally, the third axis brings out groups 3 and 4 (Online Resource 5).

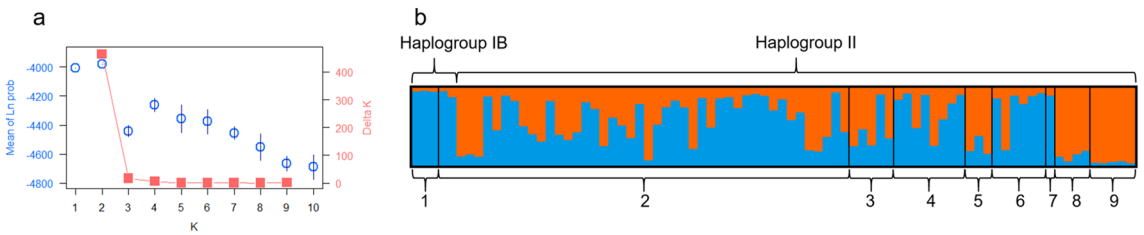


Fig. 7 Genetic assignation of *Caretta caretta* (N = 81) using STRUCTURE. **a** curves of the likelihood Ln(K) and their standard deviation (in blue) and the delta K calculated by the Evanno's method (in salmon) as a function of K (number of clusters); **b** barplot showing the structure of the 81 samples of *C. caretta* for K = 2. 1: individuals with an unknown size belonging to the haplogroup IB; 2: juveniles; 3: females smaller than 60 cm CCLmin; 4: females measuring at least 60 cm CCLmin; 5: males smaller than 60 cm CCLmin; 6: males measuring at least 60 cm CCLmin; 7: adults with an unknown size; 8: samples from the nesting event in St-Aygulf; 9: Samples from the nesting event in Villeneuve-lès-M

Discussion

This study was intended to characterise the population structure and composition of a new large sampling of loggerhead turtles from stranding and bycatch events reported from the Gulf of Lion, Provence and Corsica (French Mediterranean façade). In this respect, our sampling furnished supplementary data that are complementary to the study of Laurent et al. (1993), Carreras et al. (2007), Clusa et al. (2013), Garofalo et al. (2013), Splendiani et al. (2017) and Tolve et al. (2018) which concerned other areas of the Mediterranean Sea.

Mitochondrial *versus* nuclear diversity

The mitochondrial analysis highlighted that loggerhead turtles frequenting the French coasts of the Western Mediterranean Sea belong to two of the three major lineages currently described in this species. Haplogroups IB and II include animals from Atlantic and Mediterranean areas (Shamblin et al. 2014) whereas haplogroup IA represents turtles from the Pacific Ocean (Nishizawa et al. 2014). The haplogroup II is more widely represented in the Mediterranean Sea and 95.9% of our specimens are included in this group. Among the 12 haplotypes evidenced for the long fragment of the mitochondrial control region, CC-A2.1 characterizes 86% of the individuals and is widely distributed all along the French coasts of the Gulf of Lion, Provence and Corsica. A predominant CC-A2.1 is also observed in western (Spain, Baleares; Carreras et al. 2006), central (Italy, Malta; Garofalo et al. 2013) as well as eastern (Greece, Crete, Turkey, Cyprus, Lebanon, Israel; Carreras et al. 2007) Mediterranean basins. For the haplogroup II, five haplotypes specific to the Mediterranean (CC-A2.8, CC-A2.9, CC-A6.1, CC-A31.1 and CC-A32.1) and four haplotypes common to Atlantic and Mediterranean basins (CC-A2.1, CC-A3.1, CC-A10.4 and CC-20.1; Online Resource 2) were evidenced along the French Mediterranean façade. Haplogroup IB that mainly represents animals from the Atlantic (Shamblin et al. 2014) is represented in our sampling by only three haplotypes including seven turtles (Fig. 3).

Whatever the size of the control region analysed (241 bp and 683 bp) lower haplotype and nucleotide diversities are observed in the haplogroup II (Mediterranean and the Atlantic Ocean) as compared to the haplogroup IB (Atlantic Ocean). This result is due to the presence of haplotype CC-A2.1 in the haplogroup II characterizing 86% of our samples. This haplotype is found in high frequency, not only in the Mediterranean Sea and Northern Atlantic Ocean (including Cape Verde) but also in South Africa (Indian Ocean). This distribution suggests that all these populations shared once the same distribution area (likely a refugium) from which rapid colonization of the North Atlantic and the Mediterranean Sea was accompanied by a reduction of the genetic diversity found in the ancestral populations (Bowen et al. 1994; Bolten et al. 1998). In the Mediterranean Sea, the majority haplotype CC-A2.1 was most likely one of them, thus explaining the genetic structure and the low diversity observed. Moreover, the star-like shape of the network as well as the BSP analysis indicate a strong population expansion for the haplogroup II, a result in agreement with previous studies (Clusa et al. 2013; Reid et al. 2019). According to our BSP analysis, the beginning of the expansion phase is dated at about 130 kyrs [95% HPD: 90–180] that is during the interglacial Riss-Würm. This molecular estimation is older than the 65 kyrs (20–200 kyrs) obtained by Clusa et al. (2013) although both studies agree on the fact that *C. caretta* colonised the Mediterranean basin during the upper Pleistocene (12–130 kyrs), that is well before the Last Glacial Maximum (LGM, 18–20 kyrs). These results markedly contrast with the recent study of Reid et al. (2019) which dated the expansion at about 11–12 Kyrs (5–50 kyrs), that is after the LGM. Such a discrepancy can be explained by the different dating methods or more surely by the discordant rates of mutation that have been used in the three studies. Moreover, the difficulty to estimate molecular rates of evolution has become more complicated since the recent study of Tikochinski et al. (2020) which revealed extensive heteroplasmy of the mitochondrial control region for *Chelonia mydas*. As heteroplasmy increases the rate of change in genetic diversity, estimations of mutation rates and thus of divergence times might have been overestimated. In conclusion, if the expansion of the Mediterranean lineages of *C. caretta* seems to be confirmed, the timing of this expansion (post-LGM or more ancient) is still a matter of debate pending accurate estimations of the mutation rate.

By contrast with the strong genetic structure observed with the mtDNA, analyses of nuclear DNA (13 microsatellite loci) revealed little population structure among the 81 genotyped individuals. Indeed, the nuclear differentiation between the two mitochondrial haplogroups IB and II does not appear clearly in the STRUCTURE analysis, a result that is supported by their important overlapping in the PCA (groups 1 and 2 in Online Resource 5). From the microsatellite analysis, the main structure observed concerns the differentiation of the embryos (see Fig. 7 and Online Resource 5) coming from the two nests at the beaches of Villeneuve-lès-M. (Gulf of Lion) and St-Aygulf (Provence). If the PCA confirmed this distinction (see groups 3 and 4 in Online Resource 5), this analysis also clearly indicated that both nesting sites are genetically different, not only with respect to individuals belonging to haplogroups IB and II

but also between them. Although Atlantic and Mediterranean individuals are sharing the same foraging grounds along the French coasts, our results revealed a lower population genetic structure in nDNA assays relative to mtDNA, thus indicating a significant gene flow between individuals over generations. Previous studies (Bowen et al. 2005, Monzón-Arguëllo et al. 2010, Garofalo et al. 2013) on the genetic structure of *C. caretta* came to a similar scenario: the mitochondrial marker brought out a deep structuration resulting from the colonization of the Mediterranean Sea whereas microsatellite analysis did not reveal any structuration in their sampling, even between colonies differentiated by maternal markers. This result is often interpreted as a sex-biased gene flow resulting from opportunistic mating by males during their migration phase inversely to females that are more philopatric or faithful to successive breeding beaches. Such a hypothesis was, however, recently challenged by the study of Clusa et al. (2018) suggesting that both females and males would exhibit philopatric behaviour. Moreover, according to our MSA analysis (see next paragraph) our sampling is mostly issuing from Greece, Crete and Western Turkey which have been shown to belong to the same genetic cluster (Clusa et al. 2018). It is therefore not surprising not to identify any genetic structuring among our samples. It is clear however that a better understanding of the respective role of males and females in population delineation will benefit from using more powerful molecular markers, such as SNPs, issuing from high-throughput sequencing (Komoroske et al. 2017) as well as additional samples from at least the main spawning beaches in both Atlantic and Mediterranean basins.

Origin of loggerhead turtles from the Gulf of Lion, Provence and Corsica

According to the MSA analysis, less than 10% of loggerhead turtles (6% for adults and 8.5% for juveniles) frequenting the French Mediterranean coast originated from the Atlantic Ocean. These individuals have been assigned mostly to Florida and Bahamas rookeries. If the MSA analysis estimated that 6% of adults would have an Atlantic origin, it can be noted that no adult is characterized by a pure Atlantic haplotype (such as CC-A1) whereas three juveniles carried two haplotypes (CC-A1.1 and CC-A1.3; Fig. 4) that are characteristic of Atlantic rookeries. These results suggest that only a few adults as well as juveniles issuing from Atlantic are frequenting the Gulf of Lion, Provence and Corsica areas. Indeed, the study by Clusa et al. (2014) indicated that the majority of juvenile loggerhead turtles from the Atlantic are confined to the Algerian basin (about 60%) and to a lesser degree (between 5 and 20%) in other Mediterranean basins. If the proportion of Atlantic turtles identified in the French Mediterranean façade falls in these estimations, this is a lesser proportion than the 20% recognized in the Catalano-Balearic and Tyrrhenian Seas (Clusa et al. 2014).

About 90% of the loggerhead turtles frequenting the French Mediterranean coast are likely originating from the Mediterranean Sea. Major contributions would be from Greece (36% and 56% for adults and juveniles, respectively), Crete (12% and 18%) and Western Turkey (14% and 4%). Either for adults or juveniles, major contributors are the biggest colonies from the north-eastern Mediterranean, such as Greece or Turkey. The Calabria rookeries in Italy, the closest in the geographic distance to the French Mediterranean façade contributed only 3.1%. The absence of Calabria contribution to the Adriatic turtle composition was attributed to the small size of the Calabrian rookery combined with opposite marine currents preventing migration in the Adriatic Sea (Tolve et al. 2018). Because of the existence of favourable coastal currents (see below), only the first argument could explain the absence of Calabrian turtles on the French Mediterranean coasts. We also did not evidence input from Libya (Misrata and Sirte rookeries), while these rookeries contributed to juveniles observed in the Catalano-Balearic, Tyrrhenian and northern Ionian basins (37%, 47% and 70%, respectively; Clusa et al. 2014). Finally, as previously observed (Monzón-Arguello et al. 2010, Clusa et al. 2014), we did not identify contribution of the Cape Verde rookery in individuals frequenting the French Mediterranean shoreline.

The French Mediterranean façade is monitored during the Marine Megafauna Aerial Survey (SAMM) campaigns (Pettex et al. 2014), and sea turtles larger than 20–30 cm can be detected in the first 2–3 m below the water surface. The SAMM campaigns have confirmed the presence of sea turtles with important concentrations, especially juveniles, along all the French Mediterranean coasts (*e.g.* on the eastern coast of Corsica and at the Rhône delta, Darmon et al. 2017). Loggerhead turtles off the French shorelines are likely carried from eastern to the western Mediterranean by sea-surface currents (such as the Liguro-Provencal current, review in Mansui et al. 2020), as previously proposed for juveniles in the western Mediterranean basin with both genetic (Carreras et al. 2006; Clusa et al. 2014; Cardona and Hays 2018) and direct (marking and telemetry; Casale et al. 2007; Revelles et al. 2007, Zbinden et al. 2008, Schofield et al. 2010) data. The presence of juveniles identified as originated from Greece, Crete, Turkey, Lebanon and Israel is in agreement with the global water circulation at the Mediterranean scale (Mansui et al. 2020). However, increasing the data on the origin of individuals (genetic and direct observations by marked individuals) and on water circulations at small scales will allow a better understanding of the complex course of juvenile loggerhead turtles originated from and outside Mediterranean waters. Moreover, even if the mtDNA database has clearly been improved with respect

of haplotype recording from Mediterranean rookeries, MSA resolution is limited by the high frequency and ubiquity of haplotype CC-A2.1. Here again, new types of molecular markers might help reaching a more powerful identification of turtle's origin in the future (Komoroske et al. 2017; Hamabata et al. 2020).

Sporadic nests occurred on the French Mediterranean coasts and samples were collected at the two most recent nesting sites in Provence (St-Aygulf in 2016) and Gulf of Lion (Villeneuve-lès-M. in 2018). Such sporadic nesting observed in other parts of the western Mediterranean (Spain and Italy) are characterized by haplotypes found both in Atlantic and Mediterranean rookeries, thus suggesting independent and long-distance colonization of the western Mediterranean (Carreras et al. 2018). Unfortunately, only the majority haplotype (CC-A2.1) has been identified among the 9 sequences obtained for the control region from these two nesting events in France. As this haplotype is found in nearly all rookeries, the possible origin of turtles is multiple: from Atlantic (Florida and Bahamas) to multiple rookeries from the Mediterranean (e.g. Greece, Turkey, Italy) or even from Cape Verde or South Africa. By contrast, the microsatellite analysis indicated that individuals from nests are clearly differentiated from other turtles analysed (Fig. 7 and Online Resource 5). This could argue for an origin from rookeries not represented in our sampling (such as Libya) or outside the Mediterranean Sea (*e.g.* diverse Atlantic places), but testing this hypothesis will necessitate including more representatives of turtles outside the Mediterranean Sea. In the future, it will be thus interesting to pursue the analysis of egg-laying sites occurring on the French shoreline to better assess the origin of these new colonizers, a data that will have some importance for the definition of management units (Shamblin et al. 2014).

Conservation issues

On the basis of diverse information, such as geographic distribution, monitoring localisation, number and localisation of nesting sites, genetic structure inferred from mitochondrial (control region sequences) and nuclear (microsatellites) DNA, foraging areas, Wallace et al. (2010) defined several Regional Management Units (RMUs) at the worldwide scale for *C. caretta*. The Mediterranean Sea represents one separated RMU although, it is clear that the Mediterranean is also frequented by individuals from two other independent RMUs: the northwest Atlantic and the northeast Atlantic (Monzón-Argüello et al. 2010, Wallace et al. 2010). In the Mediterranean RMU, Shamblin et al. (2014) recognized seven Management Units: Calabria (Italy), western Greece, Crete, western Turkey, eastern Turkey, eastern Mediterranean and Tunisia/Libya. These MUs were defined on the basis of haplotype distinctiveness (private haplotype) and genetic proximity of eastern Mediterranean rookeries. In this context, the Gulf of Lion-Provence-Corsica and more generally the western Mediterranean Sea was not considered as a possible MU. However, the frequentation of the western basin by high densities of juveniles and the recent nesting in French, Italian and Spanish coasts increase the importance of this region for the conservation of Mediterranean loggerhead turtle populations.

The Gulf of Lion is considered as an important feeding area, notably for juvenile turtles in the migration phase (Garofalo et al. 2013). Juveniles represent about 85% of turtles frequenting the French Mediterranean coasts, and they are facing classical threats, such as bycatch (mainly by trawling in winter and gill-netting in summer and maritime traffic, Sacchi et al. 2020). Many juveniles are concentrated in front of the Rhône delta (Pettex et al. 2014), the main Mediterranean river in term of nutritive input (Raimbault et al. 2009). These turtles likely follow the main east–west current (Northern Current; Mansui et al. 2020) and thus cross the sanctuary Pelagos, a large marine protected area (MPA) which covers 87 000 km^2 between northern Sardinia, Provencal coasts and north-western coasts of Italy. There is no MPA directly in front of the Rhône River delta, the closest being the Parc Marin de la Côte Bleue or the Parc National des Calanques respectively located about 20 and 46 km (10.8 and 24.8 mi) west of the delta. However, the impact of anthropogenic activities on juvenile survival is lacking in and outside these protected areas. In the Mediterranean, 7.14% are covered by MPAs or other effective conservation measures but only 0.04% corresponds to strong protection zones (fishing limitation, traffic reduction, etc.; MedPAN 2016). As in other parts of the Mediterranean Sea, bycatch in fishing gear is the main anthropogenic threat at sea for loggerhead turtles, and "the intensity of conservation initiatives aimed at mitigating this threat is very low" (p. 256, review in Casale et al. 2018).

Occasional nesting sites are found in the western Mediterranean basin, in Spain and Italy (Carreras et al. 2018), particularly in the South Tyrrhenian coasts where nests are regularly observed since 2012 (Maffucci et al. 2016). On the Gulf of Lion and Provence shoreline, three sporadic nesting sites have been observed since 2006 in sandy beaches (localities k, af and ag in Fig. 1) which are the northernmost latitudes observed for loggerhead turtle nests in the Mediterranean Sea (Senegas et al. 2009). In Corsica, eastern beaches that present favourable nesting environments were regularly frequented until the 1940s and are again used since 2014 (Delaugerre and Cesarini 2004; Gérigny et al. 2020). A combination of factors could explain these recent nesting events, such as the increase of sea level and temperature associated to decades of protection and conservation measures especially in eastern Mediterranean rookeries (Casale et al. 2018) which would contribute to produce new

colonizers. A process of colonisation would be underway from distant eastern nesting beaches (e.g. from Greece as deduced from the presence of the haplotype CC-A32.1) but also from long-distance dispersal across oceans (Carreras et al. 2018, this study). Thus, the conservation challenges for these western Mediterranean nesting sites are the highly artificialized coasts and strong human frequentation (and thus disturbance) on the remaining sandy beaches. As in many rookeries, egg viability and post-hatching survival from sporadic nests remain unknown but protection measures have already been implemented. Current actions conducted thanks to Non-Governmental Organisations (such as the French Mediterranean stranding network and rescue centres) are the surveillance of nesting events, physical protection (barriers) of nests, tissue sampling for acquiring more knowledge on the origin of nesting turtles, and public awareness on the presence of these marine organism along the coasts.

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Availability of data and material Our manuscript has data included as electronic supplementary material.

Declarations

Conflict of interest All authors declare no conflict of interest.

Ethics approval Loggerhead turtles are strictly protected in France (national decree of October 14, 2005) and the sample collection was allowed by the decree of December 31, 2012 on the renewal of the environmental protection approval of the Société Herpétologique de France (SHF), and the decree of October 24, 2016 on the collection of biological data in the event of sea turtle stranding or bycatch on French metropolitan coasts (NOR: DEVL1500415N) for the mainland sea turtle observatory program of the National Museum of Natural History (MNHN).

Consent to participate All authors made substantial contributions to the paper and agree with the contents of the manuscript.

Consent for publication All authors approved the version to be published and its submission to *Marine Biology*.

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